"Pharmacognostic Phytochemical studies and the Effects on Angiogenesis, TNF- α Inhibition of the Leaves of *Justicia gendarussa Burm*. f"

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CERTIFICATE

This is to certify that the dissertation entitled **"Pharmacognostic Phytochemical studies and the Effects on Angiogenesis, TNF- α Inhibition of the Leaves of *Justicia gendarussa Burm. f*"** was done by **Mrs. B.Umamaheswari** in the Department of Pharmacognosy, Madurai Medical College, Madurai – 20, in partial fulfillment of the requirement for the Degree of Master of Pharmacy in Pharmacognosy, under my guidance and supervision.

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(Dr. L.SUSEELA)

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CONTENTS

INTRODUCTION

Herbal Renaissance

Herbs are staging a comeback and herbal “Renaissance” is happening all over the globe. The herbal products today symbolise safety in contrast to the synthetics that are regarded as unsafe to human and environment. The synthetic products of the modern age surpassed their importance, for a while. However, the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security.

Medicinal plants are inextricably inter-twined with the rich history, culture and culinary tradition of India. India has a rich and glorious ethanomedical heritage. It is reported that 4639 ethnic committees who lived in different regions of India use locally available medicinal plants of treat various ailments, based on their rich and varied folk knowledge.

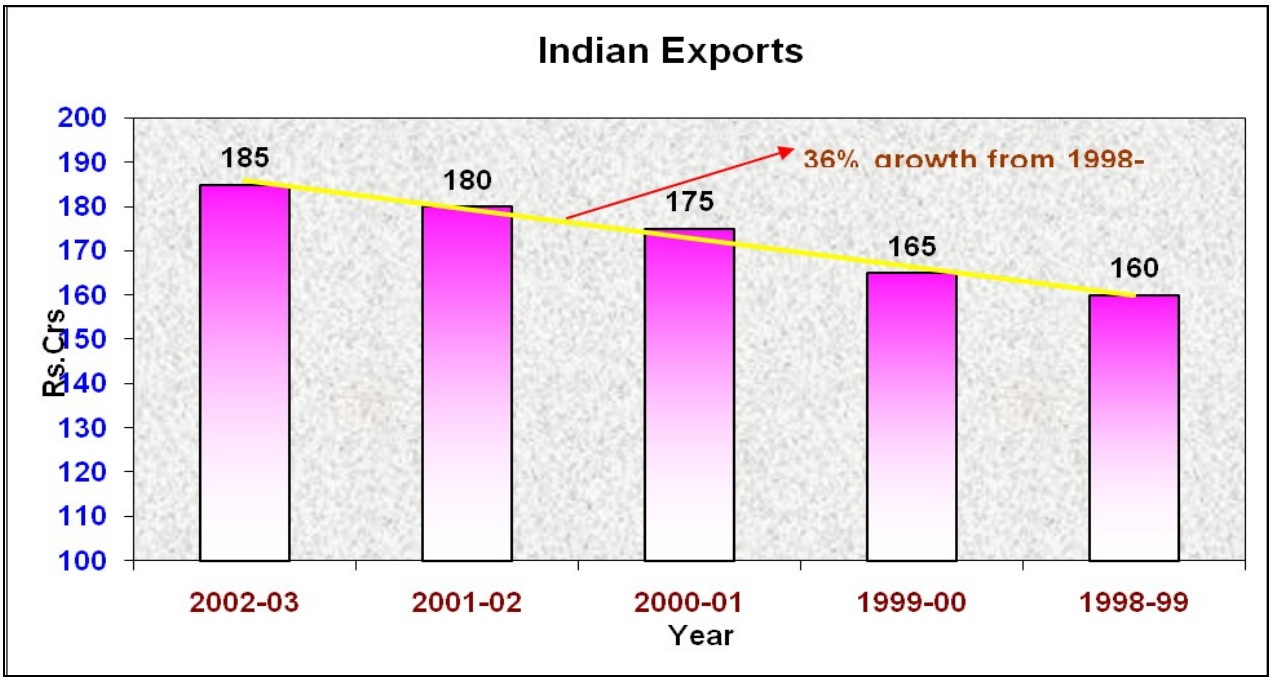
Over three-quarters of the world population relies mainly on plants and plant extracts for health care. More than 30% of the entire plant species, at one time or other were used for medicinal purposes.

It is estimated that world market for plant derived drugs may account for about Rs.2, 00,000 crores.

Herbal market a view

Presently Indian contribution is less than Rs.2000 crores. Indian export of raw drugs has steadily grown at 36% to Rs 185 crores in 2002-2003 from 160 crores in 1998-1999. The annual production of medicinal and aromatic plants raw material is worth about Rs.300 crores. This is likely to touch US$

1350 by the year 2010 and US$10 trillion by 2050. It has been estimated that in developed countries such as U.S, plant drug constitute as much as 25% of total drugs, while in fast developing countries such as china and India, the contribution is as much as 80%.Thus, the economic importance of medicinal plants is much more to countries such as India than to rest of the world**.**



Biodiversity and biotic zones

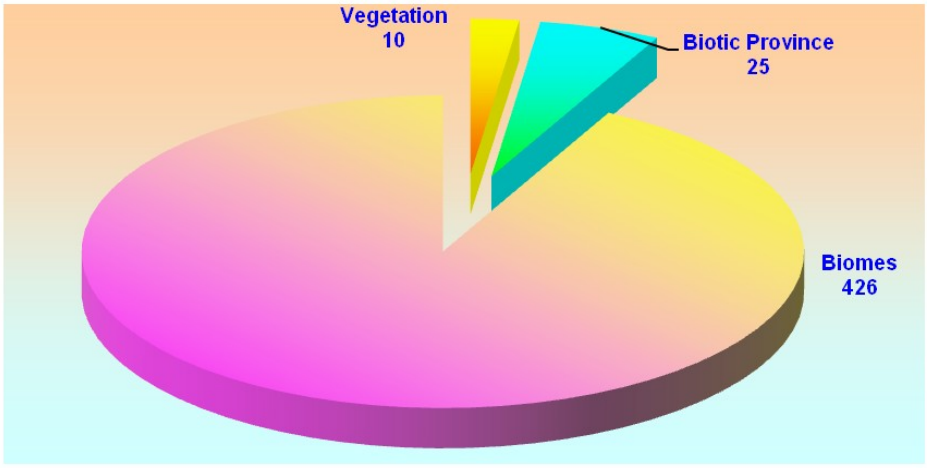
Of the 2, 50,000 higher plant species on the earth, more than 80,000 are medicinal. India is one of the world's 12 biodiversity centres with the presence of over 45,000 different plant species. India's diversity is unmatched due to the presence of 16 different agro-climatic zones.

10---- Vegetation zones.

25----Biotic provinces.

426—Biomes (Habitats of specific species)

Of



these about 15,000-20,000 plants have good medicinal value. However, only 7000-7500 species are used for their medicinal values by traditional communities. In India, drugs of herbal origin have been used in traditional systems uses about 700 species, unani 700, siddha 600, Amchi 600 and modern medicine around 30 species. Traditional systems of medicine continue to be widely practised on many accounts.

Population rise.

Inadequate supply of drugs.

Prohibitive cost of treatments.

Traditional medicinal practices

The uses of traditional medicinal plants in relieving symptoms of disease and curing various infections date back many centuries. In recent years, considerable interest has developed in Asian countries in the collection and extended use of indigenous and introduced plants for medicinal purposes.

Knowledge of medicinal plants has been the key for the survival of the ethnic groups who live in the interior. Peoples who live far from towns and in forest still rely on traditional cures handed down to them through the generations. They use these plants in one form or another to cure or alleviate a variety of ills, for example, toothache, stomach-ache,fever,common cold,pain,malaria etc.,

Side effects of several allopathic drugs and development of resistance to currently used drugs for infectious diseases. Ayurveda, siddha, unani and folk (tribal) medicines are the major systems of indigenous medicines. Among these systems, Ayurveda is the most developed and widely practiced in India. Today this system of medicine is being practised in countries like Nepal, Bhutan, Srilanka, Bangladesh, and Pakistan.

The U.S Government has established the “office of Alternative Medicine” at the National Institute of Health at Bethesda and its support to alternative medicine includes basic and applied research in traditional systems of medicines such as Chinese, Ayurvedic etc.,

Impact of Globalisation on Herbal Medicine

Globalisation in its true effect appears to favour the West and disfavour the rest. India is currently confidently handling the globalisation process. Medicinal plants have started playing a vital role in Indian economy.

Don’t imitate, innovative is the thumb rule in the modern herbal drug industry.

The medicinal plants find application in pharmaceutical, cosmetic, agricultural and food industry. The use of the medicinal herbs for curing

disease has been documented in history of all civilizations. Before onset of synthetic era, man was completely dependent on medicinal herbs for prevention and treatment of diseases. With introduction of scientific procedures the researchers, were able to understand about toxic principles. Recently research has supported biological activities of some medicinal herbs. Cancer, HIV and tuberculosis are such segments where researchers are expecting new molecules from herbs that can provide us with tools for fighting this dreaded diseases.[1]

In recent years there is a great deal of interest in developing agents to control damage induced by reactive oxygen species(ROS) and angiogenesis in biological system. Since these can contribute to the etiology of diseases such as cancer, liver diseases, atherosclerosis, respiratory diseases and inflammatory response disease syndrome. As plant produces lot of antioxidants to control the oxidative stress , they can represent a source for new compounds with antioxidant activity.Uncontrolled angiogenesis is a major contributor to a number of disease states including arthritis , diabetes-related blindness,psoriasis, and tumour growth and metastasis. While anti-angiogenic compounds would be useful in treating disease having uncontrolled angiogenesis, proangiogenic compounds are needed in wound healing and may be useful in minimizing tissue damage following ischemia damage from heart attacks or stroke.So to bridge the drug development gap antiangiogenesis is a new weapon. Inhibition of angiogenesis is a prime target afflictions such as growth of solid tumour, arthritis, and inflammations.[2]

Anti-Angiogenic Drugs and Natural products

Natural products are currently exploited to target tumour angiogenesis. A vast array of products of natural origin have been shown to have anti-angiogenic potential in preclinical models, including purified endogenous inhibitors, and exogenous compounds derived from varied species of plant.[3] ***Angiogenesis may be defined as a multistep process leading to the formation of new capillaries emerging from pre-existing blood vessel systems.*** Any imbalance in the control of this complex system may promote angiogenesis dependant diseases. Isoliquiritin and Magnoshinins isolated from Liquorice root, Magnolia Salicifolia respectively have been shown as potential inhibitors of these “deadly” processes.[4]

Angiogenesis Dependant Diseases Examples[5]

Arthritis and Herbal drugs

Arthritis is another potential disease where no satisfactory answer is present in modern medicine. Commiphora mukul (guggulosterones)Boswellia serrata (Boswellic acid)Withania somnifera (withanolides)Ruscus acueleaus (Ruscogenin)Harpagophytum procumbens (Harpagoside) are prominent

plants with anti-arthritic activity. Harpagoside is a precious constituent as it has antirheumatoid activity.[1]

Important angiogenic mediators have been demonstrated in synovium and tenosynovium of rheumatoid joints. Other angiogenic mediators such as platelet-derived growth factor (PDGF), fibroblast growth factor-2(FGF-2), epidermal growth factor (EGF), insulin – like growth factor (IGF), Hepatocyte growth factor (HGF), transforming growth beta (TGF-β) , tumour necrosis factor alpha , interleukin -1 (IL-1), IL-6,IL-8,IL-13,IL-15,IL-18, angiogenin, platelet activating factor, play an important role in angiogenesis in rheumatoid arthritis.[6]

Modulating TNF-α Signalling With Natural products

Currently, tumour necrosis factor- alpha (TNF-alpha) inhibitors from natural origins are being advanced for the treatment of inflammatory disorders. Elevated TNF-alpha synthesis has associated with the development of diabetes, septic shock, tumourigenesis, rheumatoid arthritis, psoriatic arthritis and inflammatory bowel disease. Currently, only protein- based drugs are available for the clinical inhibition of TNF-alpha activity. Small – molecule drugs that can regulate TNF –alpha levels or activity might provide a cost- effective alternative to protein – based therapeutics. [7]

Lignans and their role

Lignans are well known plant secondary metabolites, constituted by two cinnamic acid residues and are found distributed in terrestrial plants and exhibit a number of biological activities, e.x antiviral, antitumour, antibiotic activities. Mamalian lignans are structurally different from plant lignans in

having meta – substituted aromatic rings and are thought to be play chemoprotective role in humans.[8]

Reason for selecting the plant *Justicia gendarussa*

Preliminary literature survey revealed that the plant ***Justicia gendarussa*** belonging to the family Acanthaceae is known for its medicinal properties in traditional medicinal practices for various angiogenesis dependant diseases like rheumatism, inflammation, eye diseases etc.,. The presence of lignans, the naturally occurring phenolic dimers and triterpenes inspired to take this plant for our study. Lignans reported to have various biological activities including antioxidant activities..A series of dihydrobenzofuranlignans showed a pronounced antiangiogenic activity.

Aim and objective

Aim :

- To study the pharmacognostic, phytochemcial and in-vitro studies on antiangiogenesis using CAM model and TNF-α inhibitory activity of the extracts of leaves of *Justicia gendarussa*.

Objective:

- The objective of the study was divided in to 4 parts.

Part – I

Pharmacognostic studies

- Identification, collection and authentification of leaves of *j.gendarussa*.
- Detailed pharmacognostic study and quantitative microscopy and other parameters.

Part – II

Prelimnary phytochemical study.

- Prelimnary phytochemical analysis on the leaves of *j.gendarussa* powder and other different extracts.
- Indentification of the presence of β- sitosterol in the petroleum ether extract, and to get HPTLC finger prints of petroleum ether and ethanolic extracts.

Part – III

CAM ASSAY

- To evaluate the antiangiogenesis effect of ethanolic and aqueous extracts of leaves of *j.gendarussa* by using CAM model.

TNF- BIOASSAY

- To assess the effect on TNF-α activity of the ethanolic and extract of leaves of *j.gendarussa* by LPS induced TNF-α production in THF-1 cells.

ANTIMICROBAIL STUDIES

- To assess the minimum inhibitory concentration (MIC) of the ethanolic extract of the leaves of *j.gendarussa* by liquid broth method of two fold serial dilution technique.

REVIEW OF LITERATURE

Ethanomedical Uses
Entire Plant

- Anonymous, (1959) decoction of the entire plant used as febrifuge and diaphoretic and also given for stomach problems and amenorrhoea.
- In Malaya, it is used for the treatment of lunacy, debility and snake bite.[9]
- Duke J.A, et al (1985) stated that decoction of entire plant applied for bruises externally.[10]
- Yangf L.L, et al (1987) hot water extract of the entire plant used for liver disease.[11]
- Fakim G, et al, (1996) stated that entire plant decoction applied externally for haemorrhoids and fever.[12]
- Bourne L.M, (2003/2004) ***Justicia gendarussa***, known in Indonesia as besi-besi has been used there in traditional medicine for stomach ache.[13]
- Plant decoction or wine infusion (30g in one litre of water) used for intermittent fevers.[14]

Pharmacognostical studies

- Chopra R.N, et al., (1956) stated that it is cultivated through out India and found often as an escape.[15]
- The Anonymous (1959) considered to be native of China. Frequently grown on Indian gardens as border plant.[9]

- Chittendon F (1992) Propagated by cuttings and grows quickly. It should be grown in warm green house and treated like a Fuchsia. Seed – sow spring in a greenhouse. Prick out the seedlings in to individual pots when large enough to handle and grow on in the greenhouse for atleast the first winter. Plant out in late spring or early summer after the last expected frosts and give some protection over the winter. The seedlings are planted 36-48 apart. The plant requires soil P^H minimum 5.6 and maximum is 7.8.[16]
- Ghosh et al, (1972) physiology of Nitrogen nutrients in the important plant pathogens with correlation to virulens was studied.[17]
- Johnson M, et al (1997) developed a micro propagation of ***j.gendarussa*** using nodal segment on MS media with nutrients and was hardened to green house technique .[18]
- Maiti S, et al, (2001) ***J.gendarussa*** is one among the 13 plant species reported to have antivenom property and it have been used traditionally used in various tribal communities like Munda, Sava, Santal, Lodha and a non – tribal community Mahato in West Bengal. And the plant species were found to be abaundant.[19]
- Bushrabi et al, (2004) In vitro studies were conducted to induce organogenesis employing nodal, intermodal and segment explants of the medicinal plants ***j.gendarussa***. MS basal medium fortified μm. BA induced direct shoots from nodal explants ms medium supplemented with 9.05 induced calli from all explants ,which were

pronounced at the cut ends of the node .Subculture on MS medium with 8.56 μm. IAA, adventious roots developed from auxillary 75% of plants survived in the field conditions.[20]

- **j.gendarussa** an accepted name in African plant checklist and database.[21]
- It was stated that there are approximately 1019 species in this genus. Here are 10 of them.

J.penrhosiensis,J.acanthifolia,J.adhadota,Jabnormis,J.acuminata,J.adnata,J.acanthoides,J.acicularis,J.ackermanni,J.abeggi[22]

Pharmacological Review

Burkill I.H, et al, (1966) reported that ethanolic and aqueous extract (1:1) given in dog in the dose of 50.0 mg/kg showed hypotensive activity.[23]

- Yangf L.L, et al, (1987) hot water extract used orally for inflammation induced by paw immersion in hot water. [11]
- Jyothishi S.G, et al, (1992) reported the presence of lignans in ***justicia*** along with pharmacological activity.[24]
- Uawonggul N et al., (2005) reported that ***J.gendarussa*** in the concentration of 0.706mg/ml pre-incubated with Heterometrus loaticus showed 33.76% efficiency against scorpion venom cell lysis.[25]
- Arokiyaraj S et al, (2007) four medicinal plants species of kolli hills of Tamilnadu were screened for their immune suppressive Effect .The crude methanol extract of ***j.gendarussa*** (100μg/ml)showed

the highest lymphocyte inhibition(84%) by lymphocyte proliferation assay.[26]

Biological activity

- George M, et al, (1949) reported that 95% ethanolic extract showed antibacterial activity against staphylococcus aureus and escherichia coli.[27]
- Yangf L.L, et al.,(1987) ethanolic and aqueous extracts (1:1) in the concentration of 1.0 gm/ml was inactive against Glutamate-pyruvate-Transaminase inhibition in the rat liver cells.[11]

Toxicity assessment

- Burkill I.H, et al, (1966) reported that ethanolic and aqueous (1:1) extracts given in mouse intraperitoneally showed LD>50 1.0 mg/kg[23]

AERIAL PART

Phytochemistry

- Woradulayapinij et al, (2005) reported the absence of tannins in the aerial part of the plant.[28]

Pharmacological Review

- Woradulayapinij , et al (2005) studied twenty Thai medicinal plants for their HIV type 1 reverse transcriptase inhibitory activity. the water extracts of ***j.gendarussa*** showed HIV-1RT inhibition ratio (200µg/ml) higher than 90%.[28]

ETHANO MEDICAL USES:

Leaves

- Nadkarni K.M , (1954) leaves are scattered among clothes to prevent them from insects. Infusion of leaves is given in fevers .Mixed with oil used as an application to glandular swellings, and also a bath in which the leaves are saturated is very efficacious in fever cases and also in rheumatism.[29]
- Anonymous, (1959) it was stated that fresh leaves are used topically in oedema of beriberi and rheumatism.

  Juice of leaves is reported to possess the property of stopping internal haemorrhage, and used for colic pain in children. Leaves are used for the treatment of lunacy, debility and snakebite. warmed salted leaves and tender stalks in a bag used in musculo skeletal aches. Decoction of leaves is used to treat chronic rheumatism [9.]
- Burkill I.H, et al, (1966) stated that decoction of the leaves given orally for females to treat amenorrhoea[23.]
- Matsui, A.D.S, et al (1967) hot water extract of the leaves given for amenorrhoea.In the preventive treatment for premature loss of hair leaf juice rubbed in to scalp for 3 days.[30]
- Mokkhasmit, M ,et al, (1971) reported that hot water extract used as an antipyretic.[31]
- Duke, J.A, et al, (1985) stated that decoction of the leaves given orally as antiperiodic[10].

- Panthong, A, et al, (1986) crushed leaves are used as a poultice on inflamed joints[32].
- Maikhuri R.K, et al, (1993) decoction of the leaf used given orally for bodypain[33].
- It was stated that tribal communities of Anaimalai hills of T.N used leaves against snake-bite[34.]
- Grosvenor P.W, et al, (1995) stated that decoction of the leaves given for fever and leaves used externally for headache. Mixed with leaves of Mallotus paniculatus applied externally for smallpox. Hot water extract given orally for stomach ache and diarrhoea [35.]
- Grosvenor P.W, et al., (1995) stated that dried leaves used for fever,smallpox,and diarrhoea[36].
- Ilham M, et al, (1995) stated that infusion of leaves used as an analgesic,diuretic,diaphoretic and as laxative[37].
- Rahman .A.H.M.M , et al, (2008) juice made from leaves are used in asthma and paste made are used in rheumatism wound and itches[38].
- Decoction of boiled leaves used for post partum baths. It was stated that tea of leaves 50 gms to liter of boiling water given for fevers, cough ,asthma,dysuria.It was reported that decoction of leaves used as febrifuge and diaphoretic. Leaves rubbed with Crinum-asiaticum and black pepper used externally for lumbago .Leaves ground with white pepper are given to woman for 3 days every morning for amenorrhoea and for stomach troubles. Infusion

of leaves mixed with oil used as an application to glandular swellings. Infusion of leaves given internally in cephalagia, hemiplegia, facial paralysis & headache. Two ounces of fresh juice from leaves with one ounce of coconut oil used as poultice for rheumatism [14].

- Fresh juice of leaves used for earaches and mixed with mustard, used as emetic and in asthma. Juice of fresh leaves is dropped in to the ear for ear ache and into the nostril on the side of the head affected with hemi crania. Oil prepared from leaves useful for eczema[39].
- Bourne L.M, (2003/2004) **j.gendarussa** ,known in Indonesia as Besi-besi has been used there in traditional medicine for stomach ache[13].
- Julius.K it was reported that leaves used for head ache in the west coast and interior of sabah[40].
- Dr.Dukes data base stated that leaves are used as sedative, carminative, and also used for intermittent fever, jaundice[41].
- leaves mixed with honey used in the treatment of pneumonia [42].

Pharmacognostical studies

- Srivastava A , et al (2004) leaf explants of ***j.gendarrusa*** were inoculated on MS medium supplemented with 2mg 2,4-D/litre and different concentrations (0.1,0.5,1.0,2.0,3.0,5.0,7.0,10.0and 20.0 grams /litre)of one of 3 salts ,that is Nacl.KCl,Na_2So_4 . Explants were subjected to salt stress in by gradual or shock treatments, the

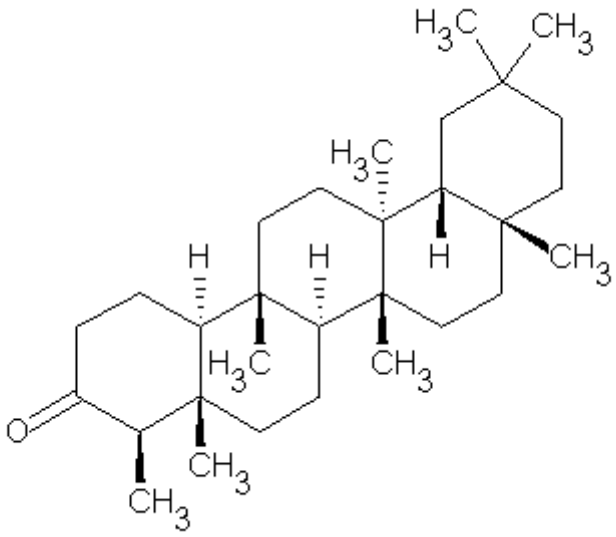
callus produced in both cases were subcultured using a diverse range of media with and without salt and it was observed that morphogenetic path that the surviving cells adopted was somatic embryogenesis[43].

- Thakur.S.S , et al ,(2006) observed the influence of day hours. , temperature and relative humidity on bee-visitation in ***j gendarussa .*** During the period of observation the air temp varied from 19°C to 28°C and relative humidity 65 to 85%. The major *Apis foragers* included *Apis dorsata, Apis mellifera and Apis cerana indica*[44].

Phytochemistry

- The Anonymous, (1959) the leaves contain a bitter and slightly toxic alkaloid [9].
- Wahi S.P , et al,(1974) chemical study of the leaf ***j.gendarussa*** [45].

- Chakravarty, et al, (1982) it was reported to contain β-sitosterol,friedelin,lupeol and four simple –o-substituted aromatic amines.Four simple O-disubstituted aromatic amines have been isolated form the leaves and characterised as 2-amino benzyl alcohol, 2-(2^1-amino-benzylamino benzyl alcohol) and their O-methyl ethers.Structures were elucidated by C ^{13} NMR.[46].



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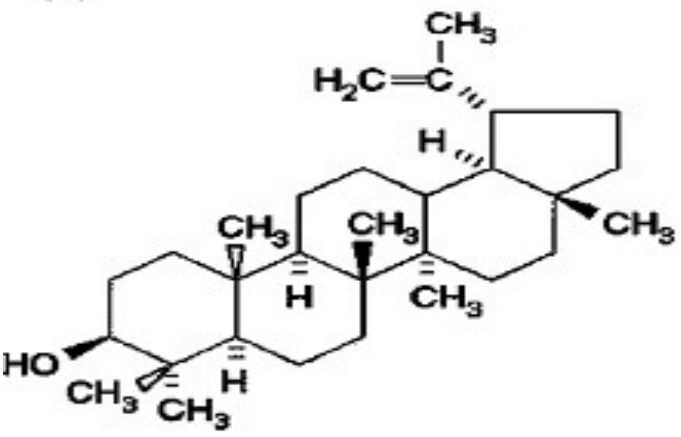

Lupeol

Friedelin

- Arbain D, et al, (1989) reported the absence of alkaloids in the leaf [47].
- Bhattacharya S , et al, (1995) it was reported that facile two step synthesis of 2 – (2^1 Amino benzyl amino benzyl alcohol) a naturally occurring amine from ***J.gendarussa***. [48]
- Oliveria A.F.M, et al (1999) it was reported that c-glycosides present in leaves and stems of ***J.gendarussa***. [49]
- Hadi S , et al, (2001) studies conducted for the presence of alkaloids from Lombok medicinal plants showed absence of alkaloids. [50]
- Mruthunjaya. K, et al, (2007) it was revealed that HPLC chromatogram of ethanolic extract of ***J.gendarussa*** measured at 275nm in the concentration range of 5mg/ml showed that it

contains constituents eluting between 2-25 minutes with major peaks at 2.68,2.833.12.317,23.37 and 23.46 min. [2]

- Gangadevi.V, et al, 2008) a novel endophytic taxol producing fungi colletotrichum gleoosporioides, isolated from the leaves of ***J*.gendarussa**[51].
- Gangadevi.V, et al, (2008) colleto-trichum gloeosporioides and phoma capitulum was isolated and determined by HPTLC [52].
- Prajogo.B, et al**,** (1994) it was reported that major compounds isolated was 6,8,-di-c-α-L-arabino-pyranosyl-4, 5,7-trihydroxy.Minor compounds 6-c- α- L arbinosyl 1,4-pyranosy flavone [53]

Pharmacological Review

- Mokkhasmit,M, et al , (1971) studied antipyretic activity in rabbits . The ethanolic and aqueous extracts in 1:1 ratio was inactive against yeast induced pyrexia in rabbit.Chronotropic effect was observed positive in ethanolic and aqueous extracts (1:1) in dogs when given in I.V and the variance active. Antispasmodic activity ,antihistamine and smooth muscle stimulant activity studied in the guinea pig ileum and the dose variance showed inactive [54]
- Ilham.m, et al (1995) ether extract of the plant in the dose of 10.0 microlitres was inactive against inflammation induced in the mouse ear [37]
- Gupta.A, et al, (1997) studied the presence of cholinesterase activity in leaves [55]

- Kepong, et al., (2004) studied evaluation of anti-inflammatory activity for piperbetle, **J.gendarussa** and cassytha filiformis using TPA induced mouse ear oedema inhibitory assay [56]
- Ratnasooriya .W.D, et al, (2007) aqueous leaf extract showed a comparable antioxidant activity to ascorbic acid and vitamin E. The antioxidant activity was dose dependant.(r^2=0.8239;p<0.05). [57]
- Mrunthunjaya .k, et al, (2007) antioxidant activity of 70% aqueous ethanolic extract of leaves of ***J.gendarussa*** evaluated by DPPH radical scavenging, nitricoxide (NO) scavenging,beta-carotene linoelic acid module system(beta CLAMS),hydroxyl(OH) radical scavenging,antilipid peroxidation and their ic50 values are of E.J were123.09+-3.01,643.0+-61.19,132.3+-6.03,68.5+-1.38 mg/ml respectively. [2]

- Ratna Sooriya.W.D, et al, (2007) it was reported that aqueous leaf extract of ***J.gendarussa*** (1500, 2000 and 3000 mg/kg) examined for oral antinociceptive action. The results showed moderate and siginificant (p<0.05) antinociceptive action when tested in hot plate,with rapid onset (2h) moderate duration of action (2-4hrs) and was dose related [57]

- Prajogo.B,etal**,** (1994) it was reported that leaf extracts showed activity as male contraceptive[51]

BIOLOGICAL ACTIVITY

- Prajogo.B,et al, (1994) reported bioactivity study on decoctions and extracts of ***J.gendarussa*** leaves. [51]

- Cavin.A, et al,(1999) dichloromethane extract in the concentration of 100.0 MCG showed inactive against fungal cladosporium cucumerinum the yeast cadida Albicans and showed inactive antioxidant activity. [58]
- Yanfg,LL,et al,(1987) ethanolic and aqueous extract (1:1) in the concentration of 1.0 MG/ML inactive against glutamate –pyruvate transaminase enzymes in the isolated rat liver cell culture.
- Ethanolic and aqueous extract (1:1) showed weak activity against carbon tetra chloride induced hepato toxicity in the isolated rat liver cells. [11]
- kumar.SN, et al (2008) eleven medicinal plants were screened for larvicidal and adulticidal activities against malarial vector,anopheles stephesi. ***J. gendarussa*** in a mixture of plant extract showed 80% and 100%.larval mortality of malarial vector and A.Stephensi [59]

- Ilham.m,et al,(1995) ether extract in the concentration of 1.0MCG/ML was inactive against Epstein –Barr virus early antigen induction [37.]

Toxicity Asessment

- Mokkhasmit,M,etal,(1971) ethanolic and aqueous extract in the dose 10.0 GM/KG given for mouse found to be inactive .The above extract given s.c in the dose 10.0 mg/KG for studying general toxic effect and it was found to be inactive. [54]
- Ratnasooriya .W.D, et al, (2007) sub chronic treatment of the aqueous leaf treatment did not elicit any over signs of toxicity stress or aversive behaviours. Aqueous extract did not induce any significant ($p>0.05$) change in haematological parameters, hepatotoxicity (in terms of SGPT levels) renal toxicity (creatinine, urea) [57]

LEAVES AND YOUNG SHOOTS

- Juice of 20 to 40 leaves or that of the young shoot extracted with water and a little wine, is administered as an emetic in coughs and asthma

  The leaves and tender stalks, put in a bag together with some salt, warmed and applied externally are useful in diseases of joints in chronic rheumatism [14]
- Gupta.A et al,(1997) studied the presence of cholinesterase activity in leaves and young shoots and it was found to be 511 pmol s^{-1} g^{-1} fr.wt[55.]

ETHNOMEDICAL USES

ROOT

- Natkarni K.M, et al.,(1954) root boiled in milk is used in chronic indigestation, dysentery, rheumatism, dysuria, fever, carbuncles, jaundice.

  It also has diuretic and diaphoretic properties as well as cooling and anodyne qualities[29]
- Duke J.A, et al, (1985) bitter root boiled with milk given for arthritis, fever, anodyne and for thrush. Boiled with milk root given as a diuretic .and given for cough [10]
- Ahmad F.B, et al,(1994) stated that decoction of the root used as a tonic. In females decoction of the root given as a post-parturition aid. [60]
- Panthong,A ,et al,(1986) crushed roots are used as a poultice on inflamed joints as an anti-inflammatory.[32]
- It was reported that 50gms of root to a pint of boling water given for diarrhoea and dysuria fevers .Stated that roots are used medicinally to treat diarrhoea. [14]

Phytochemistry

- Govindachari T.R, et al,(1969) - It was reported that β–sitosterol was not found in the roots of ***J.gendarussa*** [61]

Pharmacological Review

- The Anonymous (1959) decoction (or) the alcoholic extract of the roots produced slight paralysis in rats in doses of 1-2 gm/kg body wt[9].
- Gupta.A et al,(1997) studied the presence of acetyl cholineesterase, and it was not detected in root(>61 $pmols^{-1}$ g^{-1} fr. Wt) [53]

Toxicity Asessment

- The Anonymous, (1959) in doses of 1-2 gm/kg body wt it's antipyretic and depressant producing violent diarrhoea and eventually death[9].

Flowers

- On Vasant Panchami, Saraswathi is worshipped and in Bengal it is called the Saraswathi Pooja. They worship her with flowers specially with basaka(J.gendarussa) [62]

Part not mentioned

- Couvee , et al , (1952) reported that used to promote menses.[63]

Antiangiogenesis and TNF-α Screening Methods

- Apers S, et al, (2001) studied the angiogenesis effect on chick CAM.Albumen removed on third day, test compound mixed with agar placed on the CAM. observed under stereomicroscope the avascular zone[64].

- Vogel G.H, et al (1997) reported that cells are induced by LPS to release TNF and they are estimated photometrically at 490 nm.[65]

Antimicrobial susceptibility Test

- Antimicrobial susceptibility may be reported qualitatively, as sensitive, intermediate resistant, or quantitatively in terms of the concentration of the agent which inhibits the growth of the organism, the minimum inhibitory concentration (MIC), or that which kills it, the minimum bactericidal concentration (MBC). The concentrations of agents defining the "breakpoints" between the different categories of susceptibility are based on clinical, pharmacological and microbiological considerations.

- The three principle methods used for susceptibility Testing
    - Diffusion test
    - Minimum Inhibitory concentration
    - Break point methods

General methods for Antimicrobial screening[66,67,68]

Diffusion Method

- This method was originally designed to monitor the amounts of antibiotic substance in fermentation cultures and has also been used for obtaining biograms (Janseen et al, 1986) and for testing essentials oils.

Dilution Method

- In this method, the end point of the test is taken as the highest dilution which will just prevent perceptible growth of the test organism (MIC-value). These methods are best for assaying water soluble or lipophilic pure compounds and to determine their MIC- values, which can be recorded using this method.

Bioautographic Method

- To localize antibacterial activity on a chromatogram, has found wide spread application in the search for new antibiotics from microorganisms. The problems due to the differential diffusion of compounds from the chromatogram to the agar plate in agar diffusion technique are simplified by direct bioautographic detection on the chromatographic layer.[69]

Testing for Antimicrobial activity

- Sterilised pertiplates were preceded with 10ml of growth medium and 4 ml of inoculam in case of E.coli and S.aureus and 6.5 ml of inoculam in case of C.albicans. paper discs of 6 m.m diameter that absorb 0.1 ml of test samples (ethanol/ether) and known quantity of standard references antibiotics were used. Blank paper discs of 50 % ethyl alcohol and Ethyl ether (6 mm diameter) were kept at 5 degree for 45-55 minutes and then

incubated at 35-37 degree for 18 hours. The inhibition zone formed by the test samples were measured and compared with those of the standard reference antibiotics.[71]

Agar dilution method

- Transfer diluted bacterial suspensions to the inoculam wells of an inoculam replicating apparatus. Use the apparatus to transfer the inocula to the series of agar plates, including control plates without antimicrobial agent at the beginning and end of the series. Inoculam pins 2.5 mm in diameter will transfer close to 1µl, i,e an inoculam of 10^4 cfu/spot if bacterial suspension contains 10^7 cfu/ml and incubated at 35-37 degree in air for 18 hrs, then the zone of inhibition was readed.

Sensitivity Test

- This is perfomed by tube dilution technique A series of test tubes (16X125mm), containing 9 ml of sterile culture medium and 1ml of various concentrations of test drugs (10 tubes for each concentration) are taken and all tubes are inoculated with microorganisms to be tested and then incubated at 20-25 for 48 hours. Turbidity produced is observed there after by determining the absorbance at 530nm.

Turbidimetric or Tube assay method

- One ml of each concentration of the test drug to be screened is placed to each tube 9ml of nutrient medium previously seeded with the appropriate test organism is added. One control containing the inoculated culture medium and another blank, which is identical with the control treated immediately with 0.5 ml of dilute formaldehyde solution is used for 48 hrs.the growth of the test organism are measured by determining the absorbance at 530 nm ion spectrophotometer.[70]

Spore Germination Method

- Spore suspensions of 7 days old culture are prepared in the test compounds and standard drug for comparision of griseofulvin (in specific concentrations). A control is prepared as identical with this but without using the test compounds. A drops of spore suspension is placed on a sterilised slide and incubated in humid chamber for 12 hrs and scored the number of spores germinated to calculated the number of spores germinated to calculate the percentage of spore germination.[69]

Disc diffusion method

- This method was used for testing Antibacterial acivity. The media 25ml inoculated with suspension of experimental organism was poured in to sterilized peridishes and left to get at room

temperature. Whatman's N0:1 filter paper (7mm) were soaked in 0.2ml aqueous and alcoholic extaract as well as 10-ppm solution of Tetracycline. The filter paper discs were placed equidistantly on inoculated media and diffusion of solution was allowed to occur for 30 minutes at room temperature and incubated at 37 degree c for 24 hours and the zone of inhibition was recorded.[72]

Agar well diffusion **method**

- The inoculam suspension of each strain of bacteria was prepared to give a concentration of 107-108 bacteria/ml. The bacterial strains were grown on Trypticase 80gm broth for 24 hrs. 100µl from this culture was added to 9ml of natural saline water and 100µl from this suspension was transferred to sterile petridishes . 15ml of MH Agar was poured aseptically and the plates were kept for 15-20 mts at room temperature to allow agar to solidify wells of 4mm height and 4mm in diameter were then made in the solid medium with a metallic device and filled with the different concentrations of drug (100µl/well) and incubated at 37degree centigrade for 24 hrs and inhibitory zones was measured in m.m[73]

PHARMACOLOGICAL SCREENING OF ANTIANGIOGENIC PROFILE

SECTION - A

Introduction:

Angiogenesis (or) neo- vascularization is a complex process involving the activation, adhesion, profileration and transmigration of endothelial cells from pre-existing blood vessels. It plays a critical role in normal physiological processes, such as wound healing and also in numerous pathologies, ranging from tumor growth and metastasis to inflammation and occular disease

The major angiogenic regulator is vascular endothelial growth factor (VEGF) also named VEGF- A, which is one of the several members of VEGF family .It promotes this growth by stimulating the endothelial cells, which form the walls of the vessels and transport nutrients and oxygen to the tissue.

Vascular endothelial permeability factor also known as (VEGF) or vascular tropin, is a diffusible endothelial cell- specific mitogen and angiogenic factor that can also increase vascular permeability. It is a disulphide- linked homodimeric glycoprotein of about 34-45 k D a of 4 iso forms containing either 121, 165, 189, 206 aminoacid residues in the mature monomer.For example very recently it has been reported that emodin behaves as antiangiogenic by targeting vascular endothelial

growth factor (VEGF) ,induced endothelial cell proliferation, migration, invasion and tubule formation.

CAM ASSAY

To investigate the angiogenic effects of drugs on microvascular endothelial cells, a chick CAM assay may be employed. This will involve exposure of the CAM of Avian eggs to drugs and subsequent observation of the extraembryonic membranes in ovo using the technique of windowing. Blood vessel density and tortuosity will be photographed and evaluated using microscopic tools.

CHOICE OF EGGS

As far as possible the source of eggs should be kept constant as difference strains of eggs may vary a response to an agent. Eggs of genetic uniformity are not generally available and response variation may notable from egg to egg.

USES OF EGGS

It is in virology that the eggs has found its principal popularity as in virus works is a consequence of its susceptibility to many different viruses and its convenience as an experimental tool, especially on extra embryonic membranes. It is next most used in toxicology for it provides a convenient and sensitive indicator of toxic and teratogenic property of drugs and other chemicals .having the possible advantage without the intervention of placental membrane or reaction from maternal host .In addition to virology and toxicology its used in such fields as cancer research , endocrinology, nutrition , pharmacology and tissue

transplantation .In this last discipline particularly the CAM has been used on numerous occasions for the grafting of embryonic and neoplastic tissues and to a lesser extent adult tissues.

INCUBATION

Before incubation eggs should be held at a steady temp between 10 and 15 °c (50° and 60°) at a relative humidity of 75 to 85 %. Embryonic development starts above 23°c (80°F) .Eggs should preferably not to be stored for not more than 10 days after after which fertility may be decreased. For the incubation of eggs up to the time of incubation a commercial incubator is usually employed, which are usually electrically operated by forced air circulation by means of fan.

The eggs are turned by hand 3 times a day. Ordinary bacteriological incubator fitted with water trays, are frequently used for incubation of eggs after inoculation. This method is satisfactory when the eggs are incubated for not more than a few days after injecton

CANDLING OF EGGS (Transillumination).

Is necessary during incubation to remove clear (infertile) eggs, and those with dead embryos to mark inoculation sites and to detect dead embryos after inoculatiuon.Candling and marking of eggs must be done in a darkened room.

DEVELOPMENT OF THE EXTRA EMBRYONIC MEMBRANE

Since CAM assay make use of the extra embryonic membranes the simple account of the development of these structures are given. The structures of interest in this respect are the yolksac, chrion, amnion, and

allantois. The main structures of fertile newly laid eggs are the albumen (56%) yolk (32%) and shell and shell membrane (12%)

The albumen is an important source of water to the developing embryo and the yolk the main source of nutritive material. The first stage of the development of the embryo after fertilization is a division of the blastodisc to form a multicellular ,disc the blastoderm, which expands over the yolk. The embryo proper arises from the central part of the blastoderm, the peripheral parts are extra-embryonic and gives rise to the amniotic, chrionic and yolk-sac membranes. As the blastoderm expands the cells differentiate to form 3 layers of cells, the ectoderm (outermost), the mesoderm and the endoderm (inner most) , A horizontal cleavage of the mesoderm occurs, to form an outer (or somatic) layer, and an inner (or) splanchic layer. The ectoderm and the somatic mesoderm together form the somatopleure, and the enoderm and the for the splanchic mesoderm for the splanchopleure. The cavity between the somato plexure and splanchopleure is the coelom of which the extra-embryonic body cavity is a part. The well vascilarised splanchrnopleure spreads over the surface of the yolk to form the yolk sac. The yolk sac connected to the gut of the developing embryo by the narrow yolk stalk and the yolk thus comes to lie in a bag – like appendage from the albumen to the yolk increases the volume of the yolk up to the seventh day, after which the amount decreases until hatching . In terms of weight the yolk sac increases until the sixteenth day.

MATERIALS REQUIRED

Fertilized Chick Eggs

Egg incubator

Syringe

File/ Vial breaker

Micropipette

Stereomicroscope

EXPERIMENTAL PROCEDURE

EGG PREPARATION

Fertilized eggs (day 0) received from College of Vetenary Science, Nammakal, checked for damage and are placed in humidified atmosphere.(Egg incubator)

CANDLING

Done during incubation to remove clear (infertile) eggs and those eith dead embryos, after inoculation 2-3 ml of albumin was removed with a syringe at the lower side of the egg, and the hole was sealed with tape. Subsequently the upper part of the shell was removed, and the eggs were covered with a plastic film and incubated for another 72 hours.

PREPARATION OF TEST SUBSTANCE

Test substances were dissolved or suspended in a 2.5% agarose solution.

After gel formation the volume of agarose gel equivalent to the dose of the ethanolic and aqueous extracts of ***j.gendarussa*** were applied to the CAM, by means of a micropipette for various viscous solutions.

After 24 hours the antiangiogenic effect was observed under streomicroscope by observing the avascular zone surrounding the pellet. Agarose solution was used as blank and sunitib used as positive control.

Table:

Antiangiogenic activity of ethanolic extract of *J.gendarussa*:

Dose	Negative	Slight	Clear
10	6	0	0
25	2	4	0
50	0	0	6

Table:

Antiangiogenic activity of aqueous extract of *J.gendarussa*:

Dose	Negative	Slight	Clear
10	6	0	0
25	3	3	0
50	0	1	6
Dose	**Negative**	**Slight**	**Clear**
10	5	1	0
25	0	3	3
50	0	0	6

SECTION – B
Invitro Cytotoxicity Studies & TNF - α
Inhibitory activity of leaves of *J.gendarussa*

TNF-α

Monocytes and macrophages, non monocytic tumour cell lines, CD-4 $_{+}$ and CD- 8_{+} peripheral blood vessels T –lympocytes and some cultured T and B cell lines secrets cytokines known as Tumour Necrosis factor alpha (TNF- α) , Interleukin IL-1), (IL-6), in response to endotoxins or other stimuli. TNF- α is a soluble homotrimer of 1-7, β k- D, protein subunits. A membrane bound 26- k-D precursors form of TNF also exists.

CAUSES OF TNF- α

TNF- α causes proinflammatory actons which result in tissue injury such as degradation of cartilage and bone, induction of adhesion molecules, inducing procoagulant activity on vascular endothelial cells, increase the adherence of neutrophils and lymphocytes, and stimulating the release of platelet activating factor (PAF) from macrophages, neutrophils and vascular endothelial cells. There is evidence that associates TNF—α with infections, immune disorders, neoplastic pathologies, autoimmune pathologies and graft Vs host pathologies.

TNF—α also believed to play a central role in gram (-) ve sepsis and endotoxic shocks. Thus TNF- α has been implicated in inflammatory diseases, auto immune diseases, viral, bacterial and parasitic infections, malignancies and or neurodegenerative diseases and is a useful target for specific biologic therapy in diseases such as Rheumatoid arthritis, and Crohn's diseases. These beneficial effects are mediated in part by

reduced traffiking og inflammatory cells to the synovial and by supression of release of proinflammatory cytokines such as (IL-1).

TNF- α Inhibitory Studies

Objective: To determine the immunomodulatory activity of test drug on LPS induced TNF production in THP-1 cells

Cell lines:

1. THP-1 (Human, monocyte culture)
2. L-929 (Mouse, connective tissue)

Test extract: Ethanolic and Aqueous extract*s of Leaves of Justicia gendarussa*

Materials & Methods

1) Chemicals

3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Lipopolysaccahride (LPS), Fetal Bovine serum (FBS) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA., Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM), RPMI media, EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India.

Cell lines and Culture medium

THP-1 (Human, monocyte culture) and L-929 (Mouse, connective tissue) cell cultures were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of THP-1 and L-929 were cultured in RPMI and DMEM, respectively supplemented with 10% inactivated Fetal

Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) in an humidified atmosphere of 5% CO_2 at 37℃ until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in either 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For cytotoxicity studies, extract was weighed separately dissolved in distilled DMSO and volume was made up with RPMI supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

***IN VITRO* CYTOTOXICITY STUDIES**

Cytotoxicity studies of test extract was determined in THP-1 as per standard procedures by MTT assay given below to determine the non toxic doses of test drug fro TNF bioassay

Principle:

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl

thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used (Francis and Rita, 1986).

Procedure:

The THP-1 cell count was adjusted to 1.0 x 10^5 cells/ml using RPMI medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. Different dilutions of test extract were prepared in RPMI with 2% FBS and each dilution was added in quadruplicate wells. The plates were then incubated at 37° C for 24 hr in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted. 50 µl of MTT in PBS (2mg/ml) was added to each well and the plates were gently shaken and incubated for 3 h at 37° C in 5% CO_2 atmosphere. The plate were centrifuged at 2000rpm for 10 min and the supernatant was removed. 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of drug or test extract needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100 - \frac{\text{Mean OD of individual test}}{\text{ean OD of control group}} \times 100$$

TNF BIOASSAY

1) THP-1 cells seeded in to 40mm petridishes at a cell population 1.5 to 2 X 10^5 cells/ml.
2) To the cells different non toxic concentrations of test drug was added along with 0.1µg/ml of lipopolysaccharide (LPS) and incubated at 37^0C with 5% CO_2 for 4 h.
3) After incubation, the cell supernatant was collected, centrifuged, separated and stored at -20^0 C till further use.
4) Seeded L-929 culture into 96 well microtitre plate and incubate at 24hr at 37^0C with 5% CO_2.
5) Each sample (Cell supernatant) was diluted by two serial dilution methods (1:4) and added in to L-929 cultures and incubated at 37^0C with5% CO_2.
6) After 24hr the cell viability was determined by MTT assay as mentioned earlier.
7) The cell viability is direct indication of inhibitory properties of drugs against LPS induced TNF production in THP-1 cells.

RESULTS

Table 1: Cytotoxic effect of Ethanolic extract of *J.gendarussa* in THP-1 by MTT assay.

Test Extract	Concn. Tested in ug/ml	% Cytotoxicity	CTC_{50} in ug/ml
Justicia gendarussa	400	79.21	156.50
	200	68.57	
	100	42.31	
	50	14.22	
	25	9.71	
	12.5	0.00	
	6.25	0.00	

Table2: TNF-α Inhibitory properties of Ethanolic extract of *J.gendarussa* against LPS induced TNF production in THP-1 cells

Test Extract	Test Concn. In µg/ml	% TNF inhibition
Justicia gendarussa	100	55.95
	50	25.74
	25	14.42
Dexamethasone	200 µm	96.73
	100µm	73.71

The exhibited moderate cytotoxic properties with CTC_{50} value 156.50µg/ml. The Ethanolic extract of *J.gendarussa* exhibited good TNF inhibitory properties with 55.95% inhibition at 100ug/ml and lower inhibition at low doses.

SECTION C

ANTIMICROBIAL STUDIES OF ETHANOLIC EXTRACT OF *J.gendarussa Burm.f*

1) Determination of Minimal Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the test substances against a group of microorganisms was determined by liquid broth method of two fold serial dilution technique (Gibbons et al, 2002). In this assay, the minimum concentration of each test substance required to inhibit the growth of microorganism was determined.

For this assay, a series of assay tubes were prepared containing uniform volume (1ml) of sterile broth and equal volume of known concentration of test substance was added. The test substance in the first tube was serially diluted in twofold decreasing concentrations through the ninth tube and tenth tube was left without test substance as positive control. The tubes with the test substance i.e. from one to seventh were inoculated with 1 ml of inoculum ($1x10^6$ CFU per ml). The final concentration of test substance ranged from 1000 to 3.9 µg per ml. Solvent control and sterility controls were maintained in the experiment. The tubes were incubated at 37^0C / 28^0C for 24 / 48 h. Standard antibiotics, Pencillin, Streptomycin and Amphotericin-B were tested against gram –ve, gram +ve bacteria and fungi, respectively. The tubes were inspected visually to determine the growth of the organism as indicated by turbidity (In fact, turbidity of the culture medium is indicative of the presence of a large number of cells), the tubes in which the

antibiotic is present in concentration sufficient to inhibit bacterial growth remain clear. In experimental terms the MIC is the concentration of the drug present in the last clear tube, i.e. in the tube having the lowest concentration in which growth is not observed.

Results

Table 1: Anti microbial properties of *Justicia genoarussa* by two fold serial dilution method.

J.gendaruss Ethanolic extract	Minimum Inhibitory Concentration (MIC) in µg/ml		
	Eschericia coli	***Bacillus megaterium***	***Aspergillus niger***
Justicia gendarussa	**500**	**125**	**1000**
Standard drugs			
Pencillin	**15.6**	**-**	**-**
Streptomycin	**-**	**31.25**	**-**
Amphitrocin-B	**-**	**-**	**62.5**

The test extract exhibited moderate to poor antimicrobial activity against test organisms. The extract exhibited better inhibitory properties against *Bacillus megaterium* with MIC value 125 ug/ml. Test extract exhibited poor inhibitory activity with higher MIC values. Standard drugs exhibited potent inhibitory properties against test organisms.

METHODOLOGY

OBJECTIVE

To determine the immunomodulatory activity of test drug ***(j.gendarussa)*** the ethanolic and aquoeus extract on LPS induced TNF production in THP-1 cells.

CELL LINES

a) THP-1 (Human monocyte culture)

b) L-929 (Mouse, connective tissue)

METHOD

THP -1 cells seeded in to the cell population 1.5 to x 10^5 cells / ml.

To the cells different concentrations of test drug was added along with 0.1 µg/ml of lipopolysaccharide (LPS) and incubated at 37---c with 5% CO_2 for 4 hours.

After incubation, the cell supernatant was collected, centrifuged, separated and stored at -20---c till further use.

seeded L-929 culture in to 96 well microplate and incubate at 24hr at 37---c with 5% CO_2

Each sample (Cell supernatant) was diluted by two serial dilution methods and added in to L-929 cultures and incubated at 37--- c with 5% CO_2

After 24hr the cell viability was determined by MTT assay.

The cell viability is direct indication of inhibitory properties of drugs against LPS induced TNF production in THF-1 cells.

PHARMACOGNOSTIC STUDIES

SECTION - A

MACROSCOPICAL STUDY OF Justicia gendarussa Burm.f[9,39]

An evergreen scented, erect, branched smooth shrub, 2-4 ft high, found throughout India. .

Plant Taxonomy (Scientific Classification)[22]

Kingdom	:	plantae - plants
Subkingdom	:	viridaeplantae
Phyllum/division	:	Tracheophyta
Super division	:	Euphyllophytina
Class	:	Magnoliopsida (Dicotyledonous)
Sub Class	:	Lamiidae
Order	:	scrophulariales
Family	:	Acanthaceae
Sub family	:	Acanthoideae
Genus	:	Justicia Linnaeus
Species	:	gendarussa Burm.f
Botanical name	:	Justicia gendarussa Burm.f
Synonym[39]	:	Gendarussa vulgaris Nees
		Adhatoda subserrata Nees
		Dianthera subserrata Nees

Blanco gendarussa vulgaris Nees

Common Names[20] : Daun Rusa, Gandarusa, warer willow

Vernacular Names[67]

Malay	:	sikappar , Tolonsi
Indonesia	:	besi- besi
Philippines	:	kapanitulot
Thailand	:	chiang phraa man
English	:	Daun rausa
Chinese	:	Qin q iu
French	:	Ayapana Marron
Hindi	:	Nili - nargandi
Malayalam	:	Vatankolli
konkani	:	kalo-negundo
Bengali	:	Jagatmadan
Sanskrit	:	Nila nirgundi , Krishna nirgunda
Tamil	:	karunochi
Telugu	:	Nallanochili,Adda –saramu

Geographical Distribution[9,68]

An erect undershrub found throughout India also cultivated as a hedge plant.It is concisered to be a native of China and also distributed in Malaysia, Phillipines and Java.

Native

Native of china, also found in almost all Asian countries

Habit : Shrub

Habit and Habitat[20,39]

Chiefly found along the streams at low and medium altitudes in both primary and secondary forests, and sometimes in thickets in about in towns, rarely been planted. It occurs in India to Malaysia and in many Asian Countries.The plant prefers as if, neutral and basic soils. It grows in semi-shade.It requires moist soil (Fig -1).

Description

Leaves[39,68]

Colour	**:**	Green
Odour	**:**	Characteristic Odour
Taste	**:**	Blunt
Texture	:	Soft
Margin	:	Entire

The leaves are simple, opposite, lanceolate or linear lanceolate, 7 to 14 cms long , 1 to 2.5 cms wide and pointed at the ends .Short petioled , glabrous, dark violet green above and pale green beneath. Main nerves about 8 pairs, midrib and main nerves prominent. (Plate No-2. Fig No -1).

Flowers[68,69.]

Corolla white or rose with purple spots, clustered in the interrupted spikes. Hermaphrodite flowers having both male and female organs. (Plate No-).

Floral developement[74]

- The sequence of primordial initiation is acropetal and the primordia develop in the same order in which they appear. The floral apex has a two-layered tunica in all stages of development. There is no significant difference in the initiation of any of the floral appendages and thus all floral organs are homologous with respect to their histogenetic origin. The short calyx tube is formed by ontogenetic fusion of the bases of sepals; but the corolla tube arises partly by ontogenetic union of originally free parts and partly by zonal or intercalary growth. Each primordium receives a single procambium strand shortly after its initiation, except those of the posterior pair of stamens, which do not receive any vascular supply. This provides another example which goes against the doctrine of "conservatism of vascular bundles". The placentation in *Justicia* is parietal ontogenetically as well as anatomically

Fruit

Glabrous capsules , 0.5 in long, clavate, containing 4 seats.(Plate No:)

SECTION – B

MICROSCOPICAL STUDIES

J.gendarussa Burm.f

Collection of Specimens

The plant specimens were collected from Forest of Kariapatti,Virudhunagar District during August 2008. Care was taken to select healthy plants and for normal organs. The leaves were cut and removed from the plant and fixed in FAA (Formalin-5 ml + Acetic acid – 5 ml + 70% ethyl alcohol-90 ml). After 24 hrs of fixing, the specimens were dehydrated with graded series of tertiary-butyl alcohol. Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60^{0}c), until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

Sectioning

The paraffin embedded specimens were sectioned with the help of rotary microtome. The thickness of the sections was 10-12 µm. After dewaxing the sections were stained with toluidine blue. Since toluidine blue is a polychromatic stain, the staining results were remarkably good and some cyto chemical reactions were also obtained. The dye rendered pink color to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc., Where ever necessary, section were also stained with safranin and fast-green and potassium iodide (for starch).

For studying the stomatal morphology, venation pattern and trichome distribution, paradermal sections (sections taken parallel to the surface of leaf) as well as clearing of leaf with 5% sodium hydroxide and epidermal peeling by partial maceration employing jeffrey's maceration were prepared. Glycerine mounted temporary preparations were made for macerated/cleared materials.

Photomicrographs

Microscopic descriptions of tissues are supplemented with micrographs where ever necessary. Photographs of different magnifications were taken with Nikon labphot 2 microscope unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background.

LEAF

The leaf exhibits dorsiventral symmetry.

MIDRIB IN T.S VIEW (plate -5,fig-2)

Shape:

- Wide semicircular
- Size :950 µm vertically
- Adaxial side: flat topped,730µm wide having collenchymatus layer
- Abaxial side:semicircular 1mm wide

TRANSCURRENT PALISADE:

The palisade layer of lamina extend across the lower part of the adaxial hump from narrow transcurrent palisade.

Lower midrib:

4 or 5 outer layers of collenchyma present.

Groundtissue:

Large circular compact thin walled parenchymatous cells.

Upper Epidermis (Plate : 6)

Thick and prominent radially oblong or squarish polygonal in outline.the anticlinal walls are fairly thick slightly wavy.apo stochomatic,cells have narrow lumen.

Abaxial epidermis:

Slightly wider with thin wavy anticlinal walls.cells are amoeboid in outline.

Stomata:

Diacytic , 2 subsidiary cells lying on the opposite sides of the guard cells. Eliptical guard cells 30X20 µm in size.

LITHOCYSTS (Plate : 7)

At frequent intervals the epidermal cells become highly dilated and elongated parallel to the surface and possess narrowly cylindrical cystolith of calciumcarbonate bodies.The cells containing cystoliths are called lithocysts and subepidermal in position, 250µm long and 70µm wide. The cystolith is 210 µm long and 2µm thick, wider at one end and narrow at the other end.

TRICHOME (Plate :8)

Funnel shaped sessile glandular trichomes seated within a shallow epidermal cavity, and its fixed to a wide cell which is below the epidermal layer. This pedestal cell is circular and hylene, 450 µm in height,300µm in breadth. They are circular in surface view with 4 body cells and a short stock cells.

LAMINA (Plate : 9)

300 µm thick, adaxial epidermal layer, large vertically oblong cells with thick cuticle. The epidermal layer is 50 µm thick, but abaxial epidermis 30µm thick. The cells are horizontally oblong and cutically prominent.

Mesophyll

Differentiated in to adaxial zone of palisidal cells and abaxial zone of wide spongy parenchyma, having 6 layers of sperichal or lobed cells, loosely arranged with intercellular air spaces. The palisade cells are less compact 70µm in height, cylindrical with dilated upper part.

Vascular system

Shallow wide arc measuring 500 µm wide and 150 µm thick

Xylem

15 parellell lines of xylem elements with wide parenchymatous spaces in between. The xylem elements are narrow and thick walled with wide lumen.

Phloem

It occurs a thin layer of discontinuous compact cell and mixed with parenchymatous cells.

Leaf Margin

Thick blunt and slightly curved down. The inner cells of the leaf margin are circular thick walled and compact. The palisade cells are reduced in the marginal part. The lateral veins of the lamina is prominent and slightly rised beyond the surface. In this vascular bundle is collateral with a wide mass of xylem, equally wide mass or phloem strand. Vascular bundle has a parenchymatous bundle sheath of large hyaline cells.

Petiole

Studied under 2 parts. Distal and proximal parts.

Distal

Upper end has short thick blunt lateral wings

Epidermal layer

It has thick squarish cells. Some cells are modified in to lithocysts.

Outer zone

collenchymatous

Inner Zone

Parenchymatous with circular thin walled and less compa**ct.**

Proximal

Shapes semicircular with flat adaxial side no wing.

Outer zone

Collenchymatus

Inner zone

Parenchymatous

Vascular system of both distal and proxiamal

Both are similar wide bowel shaped (450 µm Wide) and (150 µmm thick)

Xylem

15 parallel uniseriate fairly wide thick walled and angular elements.

Phloem

Thin sheets beneath the xylem arc, wings of the distal portion has vascular strands 50µm in d.m.These accessory strands are collateral with small clusters of xylem and phloem.

***Powder microscopy of leaf powder of J.gendarussa* Burm. f**

Organoleptic characters

Nature: coarse

Colour: Green

Odour: Characteristic odour

Taste: Intensively bitter

The powder microscopy of the leaf powder of ***J.gendarussa*** reveals the following character

- ✓ Epidermal cells with stomata and trichome
- ✓ Diacytic type of stomata

- ✓ Glandular trichomes with epidermal cells
- ✓ Cystolith and glandular trichome

SECTION – C

QUANTITATIVE MICROSCOPY FOR LEAVES OF *J. gendarussa*

Microscopic Schedules

The vein islet number, vein terminal number, stomatal number and stomatal index were determined on fresh leaves using standard procedures.

A. Vein islet number and Vein terminal number

The term vein islet in used to denote the minute area of photosynthetic tissue encircled by the ultimate divisions of the conducting strands. The number of vein islets per sq. mm. area is called vein islet number.

Vein terminal number may be defined as the number of vein terminals present in one sq., mm. area of the photosynthetic tissue.

Determination of Vein Islet Number and Vein Termination Number

Leaf lets were cleared in chloral hydrate, stained and mounted on a slide.

A camera lucida is set up and by means of a stage micrometer the paper in divided into squares of $1mm^2$ using a 16mm objective. The stage micrometer is then replaced by the cleared preparation and the veins are traced in four contiguous squares, either in a square 2mm x 2mm (or) rectangle 1mm x 4 mm.

When counting, it is convenient to number each vein-islet on the tracing. Each numbered area must be completely enclosed by veins, and those which are incomplete are excluded from the count if cut by the top and left-hand sides of the square (or) rectangle but included if cut by the other two sides.

Ten readings for vein islet and vein termination number were recorded.

Table - 1

Vein Islet Number And Vein Termination Number Of *J. gendarussa*

Observation number	Vein Islet Number	Vein Termination Number
1	12	8
2	11	6
3	14	6
4	12	7
5	10	8
6	12	7
7	13	6
8	12	8
9	15	9
10	14	8

Range	Minimum	Average	Maximum
Vein islet Number	10	12.5	15
Vein Termination Number	6	7.3	9

Stomatal number

Stomatal number is defined as the number of stoma present in one square mm area of the photosynthetic tissues.

Method

Using fresh leaves, replicas of leaf surface may be made which are satisfactory for the determination of stomatal number and stomatal index. An approximate 50% gelatin and water gel is liquified on a water – bath and smeared on a hot slide. The fresh leaf is added, the slide inverted and cooled under a tap and after about 15-30 min the specimen is stripped off. The imprint on the gelatin gives a clear outline of epidermal cells, stomata and trichomes.

Table - 2

STOMATAL NUMBER

Observation Number	Lower epidermis
1	68
2	72
3	81
4	83
5	84
6	75
7	80
8	77
9	78
10	77

Range	Minimum	Average	Maximum
Lower epidermis	72	77.5	84

B. Stomatal Index

It is the percentage, which the numbers of stomata from the total number of epidermal cells, each stoma being counted as one cell.

I, Stomatal index = S/S+E x 100

Where S = Number of stomata per unit area

E= Number of epidermal cells in the same unit area

Determination of Stomatal Index

The procedure adopted in the determinations of stomatal number was observed under high power (45 x).The epidermal cells and the stomata were counted. From these values the stomatal index was calculated using the above formula.

Table – 3

STOMATAL INDEX

Observation Number	Lower epidermis
1	20.21
2	24.71
3	26.02
4	25.48
5	24.38
6	26.28
7	25.23
8	24.26
9	23.28
10	24.56

Range	Minimum	Average	Maximum
Lower epidermis	20.21	26.9	26.28

Determination of Palisade Ratio:

Pieces of leaves were cut off from various regions between midrib and the margin. These pieces of leaves were cleared and mounted on a slide . Camera lucida and drawing board were arranged. Then the slide was observed under high power. Four continuous epidermal cells devoid of trichomes and stomata were traced. the total number of palisade cella were counted and divided by 4.Ten such readings were taken and the average was calculated.

Table – 4

Palidade Ratio

Material	Minimum	Average	Maximum
Leaf	1.09	7.38	5.2

QUANTITATIVE SCHEDULES FOR LEAVES OF

J. gendarussa

Ash Value

The ash values were determined by using air dried powder of the leaf as per the official method.

Total ash

Two grams of the air dried leaf powder was accurately weighed in a platinum crucible separately. The powder was scattered into a fine even layer on the bottom of the crucible and incinerated by gradually increasing the temperature not exceeding 450°c, until free from carbon. Then it was cooled and weighed for constant weight. The percentage of ash with reference to the air dried powder was calculated.

Water soluble Ash

The ash obtained from the total ash procedure was boiled with 25 ml of water for 5 minutes and the insoluble matter was collected on an ash less filter paper and washed with hot water. Then it was ignited for 15 minute at a temperature not exceeding 450°c. The weight of the insoluble matter was subtracted from the weight of the total ash. The difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried powder.

Acid insoluble ash

The ash obtained from the total ash was boiled for five minutes with 25 ml of dilute hydrochloric acid. The insoluble matter was collected in a tarred sintered glass crucible. The residue was washed with hot water, dried and weighed. The percentage of acid insoluble ash with reference to the air dried drug was calculated.

Table - 5

ASH VALUES FOR THE LEAVES OF *J. gendarussa*

Observation Number	Total Ash (%)	Acid Insoluble ash (%)	Water soluble Ash (%)
1	7.61	0.50	-
2	8.62	1.02	-
3	7.23	0.55	-
4	8.41	1.02	-
5	9.32	0.65	-
6	8.86	-	7.62
7	10.03	-	6.28
8	9.44	-	6.02
9	8.78	-	6.53
10	9.99	-	5.96

Range			
Minimum	7.23	0.50	5.96
Average	8.83	0.75	6.48
Maximum	10.03	1.02	7.62

Determination of Loss on Drying

For the determination of loss on drying, the method described by wallis was followed.

One gram of the powdered leaf was accurately weighed in a tarred Petri dish, previously dried under the conditions specified in IP '96. The powder was distributed as evenly as practicable, by gentle sidewise shaking. The dish was dried in an oven at 100 – 105^0 c for 1 hour. It was cooled in a desiccator and again weighed. The loss on drying was calculated with reference to the amount of the dried powder taken.

Table - 6

PERCENTAGE LOSS ON DRYING FOR THE LEAVES OF

J. gendarussa

Material	Minimum	Average	Maximum
Leaf	6.06	7.38	8.26

EXTRACTIVE VALUES

Petroleum Ether Soluble Extractive Value

Five gram of the coarsely powdered leaf was macerated separately with 100 ml of petroleum ether in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of petroleum ether. 25 ml, of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of the petroleum ether soluble extractive value was calculated with reference to the air dried powder.

Benzene Soluble Extractive Value

Five gram of the coarsely powdered leaf was macerated separately with 100 ml of benzene in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of benzene. 25 ml, of the filtrate was evaporated to dryness in a tarred flat

bottomed shallow dish, dried at 105°C and weighed. The percentage of the benzene soluble extractive value was calculated with reference to the air dried powder.

Ethyl Acetate Soluble Extractive Value

Five gram of the coarsely powdered leaf was macerated separately with 100 ml of ethyl acetate in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of ethyl acetate. 25 ml, of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of the ethyl acetate soluble extractive value was calculated with reference to the air dried powder.

Chloroform Soluble Extractive Value

Five gram of the coarsely powdered leaf was macerated separately with 100 ml of chloroform in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of chloroform. 25 ml, of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of the chloroform soluble extractive value was calculated with reference to the air dried powder.

Ethanol Soluble Extractive Value

Five gram of the coarsely powdered leaf was macerated separately with 100 ml of ethanol in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of ethanol. 25 ml, of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of the ethanol soluble extractive value was calculated with reference to the air dried powder.

Water Soluble Extractive Value

Five gram of the coarsely powdered leaf was macerated separately with 100 ml of chloroform water in a closed flask for 24 hrs. It was frequently shaken for the first 6

hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of chloroform water. 25 ml, of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of the chloroform water soluble extractive value was calculated with reference to the air dried powder.

Table - 7

EXTRACTIVE VALUES (INDIVIDUAL SOLVENTS)

Solvents	Extractive Value (%)
	Leaf
Petroleum ether	1.02
Benzene	1.01
Ethyl acetate	0.60
Chloroform	1.92
Ethanol	1.39
Water	3.90

Extractive Values

By using solvents successively with increasing order of polarity

Five grams of the coarsely powdered leaf was extracted continuously in soxhlet apparatus for six hours individually, separately with solvents of increasing order of polarity. After six hours the solvents was removed and evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°c and weighed. The percentage of the individual solvent soluble extractive value was calculated with reference to the air dried powder.

Table – 8

EXTRACTIVE VALUES (SUCCESSIVE SOLVENTS)

Solvents	Extractive Value (%)
	Leaf
Petroleum ether	1.09
Benzene	1.68
Ethyl acetate	0.96
Chloroform	1.20
Ethanol	1.02
Water (reflux)	3.6

PHYTOCHEMICAL STUDIES

SECTION – A

The plant ***J. gendarussa*** was collected at dry forests area of Kariyapatti, Virudhunagar District in Tamilnadu during the first week of August 2009,and it was authenticated by the Taxonomist. The leaves portion was washed thoroughly and dried in shadow. The shadow dried leaves were powdered separately and then subjected to the following preliminary phytochemical studies.

ORGANOLEPTIC EVALUATION

Nature of the Powder	:	Coarse
Colour	:	Green
Odour	:	Characteristic odour
Taste	:	Blunt taste
Shaken with Water	:	Frothing occurs
Pressed in between two filter paper	:	No oily mark on the paper.

PHYTOCHEMICAL STUDIES FOR THE LEAF POWDER OF

J. gendarussa

Powdered Materials and their individual extracts obtained from different increasing polarity were subjected to the following chemical test and the results were presented in the table.

Test for Alkaloids

Various procedures to liberate alkaloids

- Powdered drug was mixed thoroughly with 1 ml of 10% ammonia solution and then extracted for 10 minutes with 5 ml methanol, under reflux. The filtrate was then concentrated.
- Powdered drug was mixed thoroughly with 1 ml of 10% sodium carbonate solution and then extracted for 10 minutes with 5 ml methanol, under reflux. The filtrate was then concentrated.
- Powdered drug was ground in a mortar for about 1 minute with 2 ml of 10% ammonia solution and then thoroughly mixed with 7 gram basic aluminum oxide. The mixture was then loosely packed in to a glass column and 10 ml chloroform was added, eluted, dried and methanol was added.
- Powdered drug was shaken for 15 minute with 15 ml of 0.1 N sulphuric acid and then filtered. The filter was washed with 0.1 N sulphuric acid to a volume of 20 ml filtrate; 1 ml concentrated ammonia was then added. The mixture was then shaken with two
- portions of 10 ml diethyl ether. The ether was dried over anhydrous sodium sulphate, filtered and evaporated to dryness and the resulting residue was dissolved in methanol.
- Powdered drug was mixed with one gram of calcium hydroxide and 5 ml of water, made into a smooth paste and set aside for 5 minutes. It was then evaporated to dryness in a porcelain dish on a water bath. 20 ml of 90% alcohol was added, mixed well and then refluxed for half an hour on a water bath. It was then filtered and the alcohol was evaporated. To that dilute sulphuric acid was added.The above made extracts were tested with various alkaloid reagents and the results were as follows.

1. Mayer's reagent	-	No cream color precipitate
2. Dragendorff's reagent	-	No reddish brown precipitate
3. Hager's reagent	-	No yellow precipitate
4. Wagner's reagent	-	No reddish brown precipitate

Test for purine group (Murexide test)

The residue obtained after the evaporation of chloroform was treated with 1 ml of hydrochloric acid in a porcelain dish and 0.1g of potassium chlorate was added and evaporated to dryness on water bath. Then the residue was exposed to the vapours of dilute ammonia solution.

No purple colour was obtained indicating the **absence** of purine group of alkaloids.

Test for Carbohydrates

- ***Molisch's test***

The aqueous extract of the powdered material was treated with alcoholic solution of α - naphthol in the presence of sulphuric acid**.**

Purple colour was obtained indicating the **presence** of carbohydrates.

- ***Fehling's test***

The aqueous extract of the powdered material was treated with Fehlings I and II solution and heated on boiling water bath.

Reddish brown precipitate was obtained indicating the **presence** of free reducing sugars.

- ***Benedict's test***

The aqueous extract of the powdered drug was treated with Benedict's reagent and heated over a water bath.

Reddish brown precipitate was obtained indicating the **presence** of reducing sugars.

Test for Glycosides

General test

- ***Test A:***200 mg of the powdered drug was extracted with 5 ml of dilute sulphuric acid by warming on a water bath, filtered and neutralised with 5% sodium hydroxide solution. Then 0.1 ml of Fehlings solution A and B were added, until it becomes alkaline and heated on a water bath for 2 mts.
- ***Test B***

200 mg of the powdered drug was extracted with 5 ml of water instead of sulphuric acid. Boiled and equal amount of water was added instead of sodium

hydroxide solution. Then 0.1 ml of Fehlings solution A and B were added, until it becomes alkaline and heated on a water bath for 2 mts.

The quantity of red precipitate formed in test A is greater than in test B indicating the **presence** of glycosides.

❋ ***Anthraquinones***

❖ ***Borntrager's test***

The powdered leaf was boiled with dilute sulphuric acid, filtered and to the filtrate benzene was added and shaken well. The inorganic layer was separated and ammonia solution was added slowly.

No color reaction was observed in ammoniacal layer indicating the **absence** of anthracene derived glycosides.

❖ ***Modified Borntrager's test***

About 0.1 gram of the powdered leaf was boiled for two minutes with dilute hydrochloric acid and few drops of ferric chloride solution was added, filtered while hot and cooled. The filtrate was then extracted with benzene and the benzene layer was separated. Equal volume of dilute ammonia solution was added to the benzene extract and shaken well.

No color was observed in ammoniacal layer indicating the **absence** of anthracene derived glycosides.

Test for cyanogenetic glylosides

Small quantity of the powdered leaf was placed in a stoppered conical flask with just sufficient water to cover it. A sodium picrate paper strip was inserted through the stopper so that it was suspended in the flask and it was set aside for 2 hours in a warm place.

No change in the colour of the sodium picrate paper was observed indicating the **absence** of cyanogenetic glycosides

❋ ***Test for cardiac glycosides***

❖ ***Keller Killiani test***

About 1 gram of the powdered leaf was boiled with 10 ml of 70% alcohol for two minutes, cooled and filtered. To the filtrate 10 ml of water and 5 drops of solution of lead sub acetate were added and filtered. The filtrate was then extracted with chloroform and the chloroform layer was separated and evaporated to dryness. The residue was dissolved in 3 ml of glacial acetic acid containing a trace of ferric chloride. To this 3 ml of concentrated sulphuric acid was added to the sides of the test tube carefully.

No reddish brown layer acquiring bluish green color after standing was observed indicating the **absence** of deoxy sugars of cardiac glycosides.

- ***Raymond Test***

To the alcoholic extract of the leaf, hot methanolic alkali was added.

No Violet color was produced indicating the **absence** of cardiac glycosides.

- ***Legal's Test***

To the alcoholic extract of the powdered drug, pyridine and alkaline sodium nitro pruside solution were added.

No blood red color was formed indicating the **absence** of cardiac glycosides.

Coumarin glycosides

A small amount of powdered leaf was placed in test tube and covered with a filter paper moistened with dilute sodium hydroxide solution. The covered test tube was placed on water bath for several minutes. Then the paper was removed and exposed to UV light.

No green fluorescence was observed indicating the **absence** of coumarin glycosides.

Test for Phytosterols

The powdered leaf was first extracted with petroleum ether and evaporated. The residue obtained was dissolved in chloroform and tested for sterols.

❖ ***Salkowski Test***

Few drops of concentrated sulphuric acid were added to the above solution, shaken well and set aside.

The chloroform layer of the solution turned red in color indicating the absence of sterols.

❖ ***Libermann – Burchard's Test***

To the chloroform solution few drops of acetic anhydride was added and mixed well 1 ml of concentrated sulphuric acid was added through the sides of the test tube and set aside for a while.

A brown ring was formed at the junction of the two layers and the upper layer turned green indicating the **absence** of sterols.

Test for Saponins

About 0.5 gram of the powdered leaf was boiled gently for 2 minute with 20 ml of water and filtered while hot and allowed to cool. 5 ml of the filtrate was then diluted with water and shaken vigorously**.**

Frothing occurred indicating the presence of saponins.

Test for Tannins

To the aqueous extract of the powdered leaf, few drops of ferric chloride solution were added.

Bluish black color was produced, indicating the **absence** of tannins.

❖ ***Gold beater's skin test***

2% hydrochloric acid was added to a small piece of gold beater skin and rinsed with distilled water and placed in the solution to be tested for five minutes. Then washed with distilled water and transferred to a 1% ferrous sulphate solution.

Formation of brown color indicates the **absence** of tannins.

Test for Proteins and Free Aminoacids

❖ ***Millon's test***

The aciduous alcoholic extract of the powdered leaf was heated with Millon's reagent.

The colour was changed to red on heating indicating the **presence** of proteins.

❖ ***Biuret test***

To the alcoholic extract of the powdered leaf 1 ml of dilute sodium hydroxide was added. Followed by this one drop of very dilute copper sulphate solution was added.

Violet color was obtained indicating the **presence** of proteins.

❖ ***Ninhydrin Test***

To the extract of the powdered drug, ninhydrin solution was added, and boiled.

Formation of violet color indicating the **presence** of Aminoacids

Test for Mucilage

To the aqueous extract of the powdered leaf, ruthenium red solution was added.

No Reddish pink color was produced indicating the **absence** of Mucilages.

Test for Flavonoids

❖ ***Shinoda Test***

A little amount of the powdered leaf was heated with alcohol and filtered. To the alcoholic solution a few magnesium turnings and few drops of concentrated hydrochloric acid were added, and boiled for 5 minutes.

Purple color was not obtained indicating the **presence** of flavonoids.

❖ ***Alkaline reagent test***

To the alcoholic extract of the powdered leaf, few drop of sodium hydroxide solution was added.

Yellow color was not formed, indicating the **presence** of flavonoids

❖ ***Zinc Hydrochloride Test***

To the alcoholic extract, mixture of zinc dust and concentrated hydrochloric acid was added.

No formation of red color indicating the **presence** of flavonoid

Test for Terpenoids

The powdered leaf was shaken with petroleum ether and filtered. The filtrate was evaporated and the residue was dissolved in small amount of chloroform. To the chloroform solution tin and thionyl chloride were added.

Pink color was obtained indicating the **absence** of Terpenoids.

Test for Volatile Oil

About 100 gram of fresh leaves, were taken in a volatile oil estimation apparatus (Cocking Middleton apparatus) and subjected to hydro distillation for four hours. No Volatile oil was obtained indicating the **absence** of volatile oil.

Test for Fixed Oil

A small amount of the powdered leaf was pressed in between in the filter paper and the paper was heated in an oven at 105°C for 10 minutes.

No translucent greasy spot occurred indicating the **absence** of fixed oil.

Table - 8

RESPONSE TO THE PRELIMINARY PHYTOCHEMICAL SCREENING FOR THE CRUDE LEAF POWDER OF *J. gendarussa*

S.NO	TEST	OBSERVATION
I.	**ALKALOIDS**	
	Mayer's reagent	-
	Dragendorff's reagent	-
	Hager's reagent	-
	Wagner's reagent	-
II	**CARBOHYDRATES**	
	Molisch's test	+
	Fehling's test	+
	Benedict's test	+
III	**GLYCOSIDES**	
	General Test	+
	Anthraquinone	-
	Cardiac	-
	Cyanogenetic	-
	Coumarin	-
IV	**PHYTOSTEROLS**	
	Salkowski test	-
	Liberman Burchard's test	-
V	**SAPONINS**	+

VI	**TANNINS**	-
VII	**PROTEINS AND FREE AMINO ACIDS**	
	Millon's test	+
	Biurett test	+
VIII	**GUMS AND MUCILAGE**	-
IX	**FLAVONOIDS**	
	Shinoda test	+
X	**TERPENOIDS**	-
XI	**VOLATILE OIL**	-
XII	**FIXED OIL**	-

Table – 9

RESPONSE TO THE PRELIMINARY PHYTOCHEMICAL SCREENING FOR THE DIFFERENT EXTRACTS OF LEAF OF *J. gendarussa*

Tests	Petroleum ether extract	Benzene extract	Ethyl acetate extract	Chloroform extract	Ethanolic extract	Aqueous extract
ALKALOIDS						
Mayers Reagent	-	-	-	-	-	-
Dragendorffs reagent	-	-	-	-	-	-
Hagers reagent	-	-	-	-	-	-
Wagners reagent	-	-	-	-	-	-
CARBOHYDRATES						
Molishch's Test	-	-	-	-	+	+
Fehlings Test	-	-	-	-	+	+
Benedicts Test	-	-	-	-	+	+
GLYCOSIDES						
General Test	-	-	-	-	+	+
Anthraquinone	-	-	-	-	-	-
Cardiac	-	-	-	-	-	-
Cyanogenetic	-	-	-	-	-	-
Coumarin	-	-	-	-	-	-
PHYTOSTEROLS						-
Salkowski Test	-	-	-	-	-	-
Libermann Burchard Test	-	-	-	-	-	-
SAPONINS	-	-	-	-	+	+
TANNINS	-	-	-	-	-	-
PROTEINS& FREE AMINO ACID						
Millons test	-	-	-	-	+	+
Biuret test	-	-	-	-	+	+
Ninhydrin test	-	-	-	-	+	+
GUMS& MUCILAGE	-	-	-	-	-	-
FLAVONOIDS						
Shinoda test	-	-	-	-	+	+
Alkaline Reagent test	-	-	-	-	+	+
Zinc hydrochloric acid test	-	-	-	-	-	-

TERPENOIDS	-	-	-	-	-	-
FIXED OIL	-	-	-	-	-	-

"+" Indicate Positive reaction "-" Indicate Negative reaction

Determination of Foaming Index

One gram of the coarsely powdered leaf was weighed and transferred to 500 ml conical flask containing 100 ml of boiling water. The flask was maintained at moderate boiling, at 80-90°C for about 30 minutes. Then it was cooled and filtered into a volumetric flask and sufficient water was added through the filter to make up the volume to 100 ml (V1)

Ten stoppered test tubes were cleaned (height 16 cm, diameter 1.6 cm) and marked from 1 to 10. Measured and transferred the successive portions of 1, 2, 3, ml up to 10 ml and adjusted the volume of the liquid in each tube with water to 10ml. Then the tubes were stoppered and shaken lengthwise for 15 seconds, uniformly and allowed to stand for 15 minutes and measured the length of the foam in every tube.

The length of the foam was less than 1 cm in every tube and hence the Foaming index in **less than 100**.

SECTION – B

THIN LAYER CHROMATOGRAPHY OF ETHANOLIC EXTRACT OF *J.gendarussa* BY USING VARIOUS SOLVENT SYSTEM

Among the various methods of separating and isolating plant constituents the "chromatographic Procedure" originated by Tswett is one of the most useful techniques of general application. All finely divided solids have the power to adsorb other substances are capable of being absorbed some much more readily than others. Thin phenomenon of selective adsorption is the fundamental principle of chromatography.

Principle

When a mixture of compound is spotted on a TLC plate the compound which readily soluble and not strongly absorbed moves up readily along with this solvent. Those which are not so soluble, and are more strongly absorbed moves up less readily to the separation of the compound.

The advancement of the TLC techniques has provided the organic chemists and biochemists a tool which combines in itself sensitivity and rapidity compound to the conventional paper chromatographic technique.

Application of substance mixture for separation

The substance mixture was taken in a capillary tube and it was spotted on TLC plated 1cm above its bottom end the start points were equally sized as for as possible.

Development of chromatogram

The plates were developed in a chromatographic tank by using a range of solvents from non-polar to polar as a mobile phase. The plates were allowed to develop ¾ of the length and then removed. The solvent front was immediately marked and the plates were allowed to dry. Then the plates were examined visually or under UV (or) sprayed with different reagents.TLC was developed using a range of solvents, among the various solvent system tested, the following combination showed 5,6 spots.

The spots were identified and Rf values were determined.

Stationary Phase : TLC Aluminium sheet precoated with

slicagel 60

F254. (merck)

Mobile phase used :Toluene : Acetonitryl

7 : 3

Detecting Agent : UV CAMAG 254 nm

The Rf value was calculated by the following formula.

$$Rf = \frac{\text{Distance travelled by solute front}}{\text{Distance traveled by solvent front}}$$

TLC of ethanolic extract of ***J.gendarussa*** by using various solvent system are tabulated here.

Table – 10

TLC OF ETHANOLIC EXTRACT OF *J.GENDARUSSA* LEAF

S. No	Mobile Phase used	Observation	Rf value
1.	Toluene : Acetonitryl 5 : 5	2 spots obtained. 1 yellow and 1 green.	0.6, 0.73, 0.77
2.	Toluene : Acetonitryl 8 : 2	4 spots obtained. 2 yellow and 2 green	0.20, 0.32, 0.73, 0.8
3.	Toluene : Acetonitryl 8 : 2	5 spots obtained. 2 yellow and 3 green.	0.2, 0.46, 0.58, 0.8

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY ANALYSIS OF *J.gendarussa* LEAF EXTRACT DEVELOPMENT OF HPTLC FINGER PRINT

The ethanolic extract of ***J.gendarussa*** leaves were applied in a concentration of 10µl using CAMAG Linomat IV sample applicator on Aluminium sheets precoated with silica gel 60 F_{254} HPTLC plates of 0.2mm thick, 5x20cm, used as a stationary phase. The plates were developed in the mobile phase, hexane : Ethyl acetate (7:3) for the ethanolic extract to a distance of 120 mm in CAMAG – Twin trough glass

chamber. Then the track was scanned using CAMAG densitometer scanner II equipped with CAMAG software © 1998 CATS 3.20", at a wavelength of 254 nm using deuterium lamp and the finger print profiles were recorded and presented in Table.

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

Instrument	:	CAMAG HPTLC system equipped with.
Applicator	:	CAMAG linomat IV sample applicator
Scanner	:	CAMAG densitometer scanner II: 951016
Software	:	CAMAG – SOFTWARE (C) 1988 CATS 3.20
Developing chamber	:	CAMAG – Twin trough glass chamber
HPTLC Plate	:	Silicagel 60F 254, Merck, pre coated HPTLC plate of 5 x 20cm, 0.2 mm thick.
Solvent systems	:	1) Toluene : Acetonitryl 7 : 3
Wavelenth	:	254 nm
Sample	:	Ethanolic extract of ***J.gendarussa leaves***

PROCEDURE

The spots of samples (10µl) were applied about 6mm from the edge of the TLC plates. The plates were developed upto 120 mm in the mobile phase.

HPTLC CHROMATOGRAM

Ethanolic extract	-	6 peaks
Petroleum ether extract	-	7 peaks.

TABLE - 11

HPTLC Profile of Ethanolic extract of *J.gendarussa leaves*

Peak Numbers	R_f	Peak Height	% of total area covered by individual peak
1	0.06	77.7	68.85
2	0.24	5.0	4.42
3	0.32	4.2	3.74
4	0.51	7.0	6.24
5	0.61	5.6	4.92
6	0.74	13.4	11.83

TABLE - 12

HPTLC Profile of Petroleumether extract of *J.gendarussa leaves*

Peak Numbers	R_f	Peak Height	% of total area covered by individual peak
1	0.06	32.2	34.37
2	0.25	6.8	7.27
3	0.32	14.6	15.56
4	0.38	5.1	5.47
5	0.64	4.5	4.77
6	0.71	15.9	16.96
7.	0.74	14.6	15.59

Results and Discussion

This dissertation covers the works on pharmacognostic, phytochemical and in vitro studies on antiangiogenesis and TNF-α inhibitory activity along with microbial studies of the leaves of ***J.gendarussa***, in an attempt to rationalize its use as single drug of therapeutic importance with multiple benefits.

Chapter I

Introduction

We discussed the importance of medicinal plants → Herbal Renaissance → Herbal market a view → Biodiversity and biotic zones →Traditional medicinal practices→Impact of globalization on Herbal medicine →antiangiogenic drugs and natural products →Angiogenesis dependant diseases examples→Arthritis and herbal drugs→Modulating TNF-α signalling with natural products →Lignans and their role →reason for selecting the plant ***J.gendarussa***

Chapter II

Review of Literature

The various literatures available were categorized under ethanomedical, pharmacological, Pharmacongostical , phytochemical, biological screening of general, aerial part, leaves and young shoots, flowers fruits for the treatment of various diseases worldwide were reviewed. Various methods for CAM assay→TNF -α Bioassay and microbial screening were also reviewed.

Chapter –III

Aim and objective

Were set to obtain the multiple benefit of antiangiogenesis , TNF-α inhibition and its dependant diseases along with antimicrobial activity without toxicity. Moreover to study ***J.gendarussa*** systematically for proper edentification and to give scientific basis to the traditional claim and the presence of lignans as one of the constituent.To find adultersntd and substituents, pharmacognostically and phytochemically.

Chapter IV
Section –A
Pharmacognostic studies

Macroscopical study including taxonomical position

→geographical distribution→the habit and habitat→description of leaves→flowers→fruit were discussed.

Photographs and line drawings were presented as an establishment of authenticity (plate:1-4, Fig:1)

Morphology of the leaf

Alternate: Simple

Size: 7 to 14 cm

Petiole : short

Shape: linear lanceolate

Margin : entire

Apex :acute-acuminate

Odour :charestic odour

Taste :blunt

Texture :soft

Midrib :wide semicircular

Adaxial :flat topped

Abaxial :semicircular

Transcurrent palisade: the palisade layer of lamina extends the lower part of adaxial hump.

Stomata: Diacytic

Lithocysts: are present

Trichome:funnel shaped sessile glandular trichomes seated within shallow epidermal cavity.

Petiole ;studied in 2 parts distal and proximal.

Powder microscopy

Abaxial epidermal cells of J.gendarussa with amoeboid outline and may wavy anticlinal walls.

Elongated cylindrical cystolith embedded in the epidermal layer (Lithocyst)

Epidermis with glandular trichome.

Diacytic stomata

Xylem, phloem.

Section –C

Deals with the quantitative microscopy in terms of microscopic and physical parameters. The results were tabulated.(Table : 1 to 4)

Parameters	Minimum	Average	Maximium
Vein islet number	10	12.5	15
Vein termination number	6	7.3	9
Stomatal number	72	77.5	84
Stomatal index	20.21	26.9	26.28
Palisade ratio			
Total ash Acid insoluble ash	7.23	8.83	10.03
Water soluble ash	5.96	6.48	7.62
Loss on drying	6.06	7.38	8.26

Extractive values- Individual solvents

Successive solvents

Solvents	
Petroleum ether	
Benzene	
Ethyl acetate	
Chloroform	
Ethanol	
Aqueous	

Chapter – V

Section –A

Deals with the preliminary phytochemcial screening of the powdered leaves and the various exrracts results were tabulated in (table: 8-9)

Constituents present	Constituents absent
Carbohydrates,glycosides,saponin,proteins and amino acids, terpenoids, flavonoids.	Alkaloids, Fixed oil, gums and mucilage, volatile oil

The presence of alkaloid was previously reported by----------But in our present study it was observed that alkaloids was absent, which may be due to soil and climatic conditions.

Section – B

Deals with the preparation of extracts and TLC and HPTLC of the ethanolic and petroleum ether extracts of leaves of ***J.gendarussa*** .Among the various solvents system tested the Toluene:Acetonotrile,70:30 showed 5 spots and 6 spots respectively. The R_f values were (Table: plate :)

HPTLC profiles of ethanolic and petroleum ether extract of *J.gendarussa* using Toluene: Acetonitrile (7:3) showed 6 peaks and 7 peaks respectively.

Chapter VI

Section –A

Antiangiogenesis activity

Deals with invitro pharmacological activity of ethanolic and aqueous extracts of leaves of ***J.gendarussa*** .The survey of ethano medical use of the leaf of ***J.gendarussa*** reveals its use in the treatment of chronicrheumatism which is a angiogenesis dependant disease. Moreover the presence of lignans which was reported as antiangiogenic was also considered for this investigation.

The results of CAM assay model showed 100% antiangiogenic activity at 50 µg/ml concentration by both ethanolic and aqueous extracts. This identify both extract as new antiangiogenic agent but gives no information on which specific steps of angiogenesis are targeted by them.

It may be by targeting Vascular Endothelial Growth Factor (VEGF) induced endothelial cell proliferation, migration, invasion, and tubule formation to get new additional insight on the features. Further investigation needed to study the exact Mechanism of Action.

Section –B

In – vitro cytotoxic activity

Cytotoxic study of the test extract was determined by THP-1 cell lines by MTT assay to determine the non toxic doses of test drug for TNF-α assay. It was found that cell Toxicity Concentration(CTC-50) for the ethanol extract was found to be 156.50 µg/ml and its shows only moderate cytotoxic activity.

Tnf –α Inhibitory activity

From the table it was observed that 56% of inhibition at 100µg/ml concentration in dose dependant manner. Though its in higher concentration its comparable to the standard drug Dexamethasone(200µm/ml) 97%

Section- c
Antimicrobial activity

Further antimicrobial assay was also performed by using ethanolic extract of leaves of ***J.gendarussa*** by two fold serial dilution method against E.Coli, Bacillus megaterium, and the fungus Aspergillus niger. The test extract exhibited better inhibitory properties against Bacillus megaterium with value 125µg/ml.But moderate activity against E.Coli 500 µg/ml and poor activity against Aspergillus niger.

Conclusion

This dissertation covers the pharmacognostic parameters of the leaves of ***J.gendarussa*** such as macroscopical, microscopical including powder analysis, quantitative microscopy and physical standards like ash values, extractive values, etc., have been studied and presented.

The tissue arrangement in the leaf was studied and presented. The presence of transcurrent palisade in the lamina and the presence of lithocysts with calciumcarbonate bodies. Funnel shaped glandular trichomes. The prelimnary phytochemcial studies revealed the presence of carbohydrate, proteins and aminoacids, glycoside, saponin and terpenoids.

HPTLC profiles of ethanolic and petroleum ether extracts were studied and presented. The Significant Antiangiogenic activity at 50µg/ml concentration by both ethanolic and aqueous extracts was studied. The inhibition of neavascularization is concentration dependant.

In the cytotoxicity study, CTC-50% of the ethanolic extract was found to be 156.50 µg/ml. The TNF-α inhibitory activity at 100µg/ml (56%) was studied. The inhibition of TNF-α activity is concentration dependant. The antimicrobial activity shows activity against bacillus ---------- and moderate activity against

The present study provide scientific basis for the ethanomedical use of this plant in chronic rheumatism and various inflammatory conditions. It is concluded that it can be optimistic that the present work proved that the leaves of ***J.gendarussa*** having therapeutic advantage to be a potential phytochemcial target in the design of a single herbal drug molecule for the treatment treatment of angiogenesis dependant diseases without cytotoxic effects with multiple benefits.

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